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(54) Title: TEST FOR PANCREATIC EXOCRINE FUNCTION			
(57) Abstract <p>It has been determined that ingested vitamin B₁₂ is bound by R protein in gastric juice, but that it is not capable of absorption in this form. In a system with a normally functioning exocrine pancreas, R protein is degraded by pancreatic proteases in the small intestine, which then allows the B₁₂ to be transferred and bound to intrinsic factor protein (IF) from which it can be absorbed. This phenomenon serves as the basis for a test for pancreatic exocrine function in which the following compositions are ingested: (1) a complex of IF and B₁₂ in which the B₁₂ is labelled; (2) a combination of another binding material, such as R protein, and B₁₂, in which the B₁₂ is labelled (in preferred embodiments the B₁₂ is labelled differently than the B₁₂ in the IF-B₁₂ complex); and (3) means for preventing R protein in the gastric juices from removing B₁₂ from the IF-B₁₂ complex, (in preferred embodiments such means are B₁₂ analogues that bind to R protein in the gastric juices; but which do not bind to IF). In preferred embodiments of the test, unbound IF is also ingested. As a part of the preferred process, steps are also taken to tie up B₁₂ binding proteins which are normally in the test subject's blood and plasma. Subsequently, the subject's excreted urine is measured for the amount of labelled B₁₂ which was originally ingested as IF-B₁₂ and for the amount of B₁₂ originally bound to the other binding material, such as R-B₁₂. The amount of B₁₂ from each source which is excreted in the urine, and the ratio between them, is indicative of the functioning of the subject's exocrine pancreas. Other aspects of the present invention include compositions containing combinations of test compositions. Yet another aspect of the invention is provided by methods of making several of the test compositions and the resulting products.</p>			

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TEST FOR PANCREATIC EXOCRINE FUNCTION

BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to methods and compositions for testing pancreatic exocrine function. More specifically, it relates to the use of the vitamin B₁₂ ingestion system as a means of measuring pancreatic exocrine function.

Prior Art

The human pancreas contains endocrine portions and an exocrine portion. The endocrine portions actually consist of a multitude of small islets that synthesize and release hormones into the blood. The exocrine portion carries the endocrine islets and makes up the bulk of the pancreas. When functioning properly, the exocrine portion synthesizes a number of digestive enzymes such as amylase, trypsin, chymotrypsin, elastase, lipase, and others. These enzymes are released into the upper part of the small intestine and are essential for proper digestion and nourishment.

On occasion the exocrine portion of the pancreas may be caused to malfunction. Such malfunction may be due to damage caused in a variety of ways, including chronic alcoholism, trauma, and pancreatic cancer. Due to the fact that the pancreas is located deep in the abdomen it is extremely difficult to evaluate clinically. X-rays and scans of the pancreas are discernably abnormal in only a relatively small proportion of patients with actual pancreatic exocrine insufficiency.

Several tests of pancreatic exocrine function are currently available. However, such tests are time consuming, cumbersome and inconvenient. One prior art test involves measuring the amount of fat in feces. This fecal fat test is based on the fact that fecal fat is increased in the absence of the pancreatic exocrine enzyme lipase which is required for the digestion and absorption of fat in the intestine. To accomplish this test the patient



must save all of his feces for 72 hours and the faces must then be analyzed for fat in the laboratory. This test is unpleasant for the patient and for laboratory personnel and, even then, it is not available at many hospitals.

5 Furthermore, this fecal fat test lacks specificity to pancreatic exocrine insufficiency, since it also provides abnormal results in a variety of intestinal diseases such as Crohn's disease and sprue.

Another prior art test, duodenal intubation, involves
10 placing a tube into the upper small intestine via the mouth and stomach and then collecting pancreatic juice which is subsequently analyzed for volume, bicarbonate and pancreatic enzymes. This test is time consuming, expensive, inconvenient and unpleasant for the patient, and again, it is
15 available at only a relatively small number of medical centers. It is therefore seen that there is presently no convenient easily applied test for pancreatic exocrine function.

It has been noted, for example, employing standard
20 Schilling tests that B_{12} is malabsorbed by about 50% of patients diagnosed to have pancreatic exocrine insufficiency. However, B_{12} malabsorption by itself is not a reliable indication of pancreatic exocrine dysfunction as B_{12} absorption is not abnormal in more than about one-half of
25 patients with such dysfunction, and further, as B_{12} absorption is also abnormal in cases of pernicious anemia and also as a result of a number of intestinal diseases.

The Schilling test, noted above, utilizes labelled B_{12} , for example, B_{12} including a radioactive cobalt isotope,
30 to measure and evaluate absorption of B_{12} by the digestive system. A modified or dual labelling Schilling test is commercially available, which uses two differently labelled forms of B_{12} , one of which is bound to intrinsic factor, and the other of which is unbound. However, such tests
35 utilizing labelled B_{12} have at no time been used to independently evaluate pancreatic exocrine function.



For many years it was believed that ingested vitamin B₁₂ was bound in the form of a complex to a protein known as intrinsic factor (IF) present in the gastric juices of the stomach. It was further believed that this IF-B₁₂ complex remained intact until it attached to specific receptors located at the end of the small intestine at which receptors the B₁₂ was unbound and absorbed into the body. It has now been determined that this model for B₁₂ ingestion is incorrect and that use of the correct B₁₂ ingestion model can now serve as the basis of accurately evaluating pancreatic exocrine function.

BRIEF DESCRIPTION OF THE INVENTION

As used throughout this application, the term "B₁₂" shall mean and include "vitamin B₁₂" and the various "cobalamin" compounds and "corrinoids" which are forms of or antecedents of vitamin B₁₂.

The present invention is based on a recently discovered model of B₁₂ absorption into the system. As already noted, it had been previously believed that ingested B₁₂ formed a complex with and was bound to the protein designated as "intrinsic factor" (IF) which is normally present in the gastric juices. It has now been determined that the mechanism for B₁₂ absorption is a two-step process. In this two-step process B₁₂ initially binds preferentially with another protein, designated as "R protein," which is also normally present in the gastric juices. This complexing of B₁₂ with R protein is overwhelming, even in the presence of IF protein. On the order of about 98 to 99% of the B₁₂ reacts with normally available R protein to form a complex, which is herein designated as "R-B₁₂." In situations in which B₁₂ is already complexed with IF, available R protein will remove the B₁₂ from the IF to overwhelmingly form R-B₁₂. After R-B₁₂ complex enters the small intestine it is then degraded by enzymes from the exocrine pancreas, such as trypsin, chymotrypsin and elastase. Following this degradation the B₁₂ can then bind with IF to form an



IF-B₁₂ complex from which B₁₂ is eventually ingested via receptors at the end of the small intestine.

It has also been recently discovered that there are vitamin B₁₂ analogues, which respond to many tests in a manner similar to B₁₂, but which do not support the same bodily functions as B₁₂. The relationship between B₁₂ and analogue B₁₂, as well as the accurate testing of B₁₂ in the presence of B₁₂ analogue is set forth in great detail in pending U. S. Patent Application Ser. No. 893,524, 10 also by Robert H. Allen and assigned to the same assignee as the present application. It has also been discovered that while R protein will complex with either B₁₂ or analogue B₁₂, that IF is specific to and will only bind with true B₁₂.

15 Discovery of the two-step B₁₂ ingestion process, of B₁₂ analogue, and of the ability of B₁₂ analogue to bind with R protein, but not with IF, serve as the basis for the present invention.

It is postulated that since B₁₂ initially preferentially binds with R protein the digestive system to form R-B₁₂ complex, and that since the R-B₁₂ complex is stable, until acted upon by enzymes produced by the exocrine portion of the pancreas, that a method of accurately determining the ingestion of initially B₁₂ bound to R reflects 20 on the availability of digestive enzymes from the exocrine portion of the pancreas. It is further postulated that the availability of digestive enzymes from the exocrine portion of the pancreas reflects on the functioning of the exocrine portion of the pancreas.

30 This theory is made the basis of the test of the present invention, which, by providing two differently labelled B₁₂ compounds and manipulating the digestive environment of the patient undergoing evaluation provides a test for pancreatic exocrine function. While the present 35 invention may simplistically be considered a "dual labelled" Schilling type test, it requires steps and materials not



normally used in a Schilling test.

For purposes of this specification a first labelled B₁₂ compound designated as "B₁₂^{*}" and a second, but differently labelled B₁₂ compound, designated as "B₁₂^{**}" are required. B₁₂^{*} and B₁₂^{**} may be, for example, radioactive (57^{Co}) B₁₂ and radioactive (58^{Co}) B₁₂. Other isotopes or means of labelling B₁₂, may also be used.

In the practice of the present invention, as a test for exocrine pancreatic function, a patient ingests, either as a single mixed composition or as a series of several compositions: (1) vitamin B₁₂^{**} bound to intrinsic factor, and hereinafter designated as "IF-B₁₂^{**}"; (2) vitamin B₁₂^{*} bound to R protein, and hereinafter referred to as "R-B₁₂^{*}"; and (3) a substantial amount of B₁₂ analogue. In preferred embodiments a fourth component containing unbound intrinsic factor, hereinafter "IF," is also included.

Subsequently, as detailed below, the amount and ratio of B₁₂^{*} and B₁₂^{**} absorbed, as assayed by measuring the amounts in the urine using the Schilling procedure is indicative of the function of the exocrine pancreas.

In the general practice of the process of the present invention the patient undergoing test is given an injection of B₁₂, or is otherwise treated to remove free binding protein from his blood, and caused to ingest IF-B₁₂^{**}, R-B₁₂^{*} and B₁₂ analogue. Subsequently his urine is collected and the amount and ratio of B₁₂^{*} and B₁₂^{**} measured. Normal exocrine pancreas function will be attributed to patients who excrete B₁₂^{*} and B₁₂^{**} in substantially the same proportion as it was ingested. Abnormal pancreatic exocrine function will be attributed to patients who excrete substantially more B₁₂^{**} than B₁₂^{*} in relation to the proportions ingested.

The following mechanism is postulated for this test. The vitamin B₁₂ injection which the patient is given binds the subject's then available binding proteins in the blood and serum so that that binding material is not available to bind with the ingested B₁₂^{*} and B₁₂^{**} samples, both of



which can thus be excreted in the urine in substantial amounts. In the digestive system the ingested B_{12} analogue binds with substantially all of the R protein naturally in the stomach, thus making the natural R protein substantially unavailable for competition with the IF- B_{12}^{**} so that the IF is not replaced with R in the stomach to form R- B_{12}^{**} .

In evaluating the results of the test of the present invention, subjects with normal pancreatic exocrine function are found to absorb and excrete significant amounts of both B_{12}^* and B_{12}^{**} in about the same proportion as the amounts of B_{12}^* and B_{12}^{**} originally ingested. This is due to the fact that the IF- B_{12}^{**} is readily available for absorption without interference from R protein (which has been bound by the ingested B_{12} analogue), while the pancreatic exocrine enzymes in the normal system cause the R- B_{12}^* to be degraded so that the B_{12}^* can then bind with IF in the system for absorption via the receptors. However, not much of either the B_{12}^* or B_{12}^{**} remains in the body, since the normal binding protein has been bound by a vitamin B_{12} injection.

Utilizing the test of the present invention, patients with pancreatic exocrine insufficiency will absorb and excrete substantial amounts of B_{12}^{**} which was initially bound with IF. However, using this test, they will absorb and excrete very little B_{12}^* . This is due to the fact that, as ingested the B_{12}^* was initially bound to R protein. Therefore, the absence or insufficiency of pancreatic exocrine enzymes to digest the R- B_{12}^* does not allow the B_{12}^* to be released from R protein for binding with IF and for absorption as IF- B_{12}^* . Thus, during the practice of the present invention, low excretion of B_{12}^* signals a lack of pancreatic exocrine enzyme in the system, and thus a malfunctioning exocrine pancreas.

In the absence of pancreatic disease, patients having intestinal diseases, which interfere with B_{12} absorption, absorb and excrete decreased, but essentially proportional



amounts of B_{12}^* and B_{12}^{**} to the amounts ingested.

Patients with both pancreatic exocrine disease and intestinal disease will also absorb and excrete decreased amounts of both B_{12}^* and B_{12}^{**} , but the amount of decrease for

5. the B_{12}^* is disproportionately greater than the amount of decrease for the B_{12}^{**} .

In preferred embodiments, the patient undergoing test is also caused to ingest unbound IF. This assures the availability of IF to bind with B_{12}^* when and if it becomes 10 available from degraded R- B_{12}^* . This assures the test against erroneous results should the patient fail to have sufficient IF in his gastric juices.

Other embodiments of the present invention include mixtures of compositions containing IF- B_{12}^{**} , R- B_{12}^* and B_{12} analogue, with and without unbound IF. Methods of making 15 IF- B_{12}^{**} and the R- B_{12}^* complex constitute yet other embodiments of the present invention.

The foregoing and other objects, features and advantages of the invention will be apparent from the following 20 more particular description of preferred embodiments of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The following is set forth as a prelude to describing the process and compositions of the present invention.

25 They provide details of certain experiments which were carried on and which led to the hypothesis upon which the present invention is based. Additionally, they provide evaluation of that hypothesis and further indicate the degree of specificity and sensitivity of the tests of the 30 present invention.

As already noted, the present invention is based upon a B_{12} absorption model which proposes that ingested B_{12} is first bound to R in the gastric juices. It is further proposed that only after pancreatic proteases act is the R- B_{12} 35 complex degraded so that B_{12} can bind with IF for absorption into the system. Based on this model normal subjects should



absorb B_{12} originating as intrinsic factor- B_{12} originating as R protein- B_{12} in equivalent amounts. Furthermore, ratios of excreted B_{12} originating as intrinsic factor- B_{12} to B_{12} originating as R protein- B_{12} should be close to the ratio at which they were ingested. Subjects with pancreatic exocrine insufficiency should absorb B_{12} originating as intrinsic factor- B_{12} normally, but should malabsorb B_{12} originating as R protein- B_{12} . Thus, if given substantially equal amounts of IF- B_{12} and R- B_{12} they should have high ratios for B_{12} originating as intrinsic factor- B_{12} to R protein- B_{12} . Subjects with intestinal disease would often malabsorb B_{12} originating as both intrinsic factor- B_{12} and R protein- B_{12} , but such malabsorption should be equivalent.

As used herein, " B_{12}^{**} " shall always designate B_{12} originating as an intrinsic factor- B_{12} complex, and "IF- B_{12}^{**} " shall designate that complex. " B_{12}^* " shall always designate B_{12} originating as R protein- B_{12} complex, and "R- B_{12}^* " shall designate the latter complex.

Materials and Methods

(^{57}Co) B_{12} (12.5 uCi/nmol) and (^{58}Co) B_{12} (4.0 uCi/nmol) were obtained from Amersham-Searle Corp., Arlington Heights, IL. The (^{58}Co) B_{12} was obtained in the form of capsules such as those utilized in the Dicopac Schilling Test Kit. Each capsule was opened and the contents dissolved in 5.0 ml of 0.15 M NaCl solution and centrifuged at 10,000 x gravity for 15 minutes at a temperature of 4°C. Then the supernatant liquid containing the (^{58}Co) B_{12} was stored at -20°C. Crystalline B_{12} was obtained from Sigma Chemical Company, St. Louis, MO., as CN-cobalamin assayed spectrophotometrically, and utilized to dilute the (^{57}Co) B_{12} to a specific activity of 2.5 uCi/nmol.

B_{12} analogue in the form of cobinamide was prepared and isolated as described by Allen et al J. Clin. Invest. 60:1381-1392, 1977, except that cobinamide and 13-epicobinamide were not separated from each other by paper chromatography.



Crystalline hog trypsin and bovine chymotrypsin were obtained from Sigma Chemical Company.

Crystalline hog elastase was obtained from P-L Biochemicals, Inc., Milwaukee, WI.

5 Pancreatic extract was obtained from Kremers-Urban Company, Milwaukee, WI., in the form of capsules designated as (KU-ZYME HP).

10 Homogenous human intrinsic factor, hog R protein, rabbit anti-human intrinsic factor serum, and human anti-intrinsic factor blocking antibodies were obtained as described previously by Katz et al, J. Clin. Invest. 53:1274-1283, 1974.

Human saliva served as the source of human R protein.

15 Human duodenal juice was collected with a dual lumen duodenal tube. The distal opening was positioned at the ligament of Treitz under fluoroscopic control with the second opening being located 12 cm proximally. Pancreatic secretion was stimulated by perfusing a mixture of essential amino acids and glucose through the proximal opening 20 at a rate of 6 ml/min and samples were collected through the distal opening. The perfusate used to stimulate pancreatic secretion contained the following; in g/liter: lysine, 5.5; tryptophan, 2.0; phenylalanine, 5.5; methionine, 8.5; threonine, 3.8; leucine, 7.9; isoleucine, 3.7; 25 valine, 6.1; and glucose, 50.

Vitamin B₁₂ binding assays were performed as described by Allen et al, J. Biol. Chem. 248: 3660-3669 (1973).

30 The transfer of (⁵⁷Co)B₁₂ from R protein to intrinsic factor was performed at pH 8.0 and was based on the precipitation of (⁵⁷Co)B₁₂ intrinsic factor with rabbit-anti-intrinsic factor antisera in 40% (NH₄)₂SO₄ as described by Allen et al, J. Clin. Invest. 61: 47-54, 1978.

35 Trypsin was assayed, as described by Erlanger et al, Arch, Biochem. Biophys. 95: 271-278, 1961, with benzoyl arginine p-nitroanilide, and the results were expressed as ug equivalents of hog trypsin which was used as the standard.



The following studies are indicative of the fact that pancreatic enzymes are required in order to transfer B₁₂ from B₁₂-R complexes to B₁₂-IF complexes.

The results of in vitro studies with human R protein and purified pancreatic proteases are presented in Table 1 and indicate that 50% of the B₁₂^{*} was transferred from human R protein to human intrinsic factor at concentrations of hog trypsin and bovine chymotrypsin of 80 and 1200 ug/ml, respectively. Hog elastase was the least effective in transferring B₁₂^{*} from R protein to IF, with only 11% transfer occurring at a hog elastase enzyme concentration of 2250 ug/ml. However, these proteases appear to act synergistically since an equal mixture of all 3 enzymes effected 50% transfer at a total protein concentration of 63 ug/ml (concentration of each enzyme 21 ug/ml).

The results of identical studies performed simultaneously with hog R protein are also shown in Table 1. At enzyme concentrations of 2250 ug/ml only 11, 4 and 0% of B₁₂^{*} was transferred from hog R protein to human intrinsic factor with hog trypsin, bovine chymotrypsin, and hog elastase, respectively. Only 6% transfer of B₁₂^{*} occurred with an equal mixture of all 3 enzymes at a total enzyme concentration of 2250 ug/ml. These results indicate that hog R protein-B₁₂^{*} is 50-100 fold more resistant to the action of pancreatic proteases than is human R protein-B₁₂^{*}.

Table 2 shows the results of similar studies performed with a freshly prepared solution of hog pancreatic extract (KU-ZYME HP) and freshly collected samples of human duodenal juice obtained before and after pancreatic secretion was stimulated by the duodenal perfusion of essential amino acids. As shown in Table 2, all three solutions caused 50% transfer of B₁₂ from human R protein to human intrinsic factor when they were present at 1 to 5% of their full strength. The sample of human duodenal juice obtained after pancreatic stimulation was approximately 5 times more potent than the unstimulated sample, while the solution of



Table 1
ABILITY OF PURIFIED PANCREATIC ENZYMES TO EFFECT THE TRANSFER OF
 $(^{57}\text{Co})\text{B}_{12}$ FROM R PROTEIN TO INTRINSIC FACTOR

Percent Transfer of $(^{57}\text{Co})\text{B}_{12}$ from Human R Protein
to Human Intrinsic Factor by Various Pancreatic
Enzymes

enzyme added (ug/ml)	trypsin (%)	chymotrypsin (%)	elastase (%)	all three (%)
0	0	0	0	0
5.5	5	0	0	5.7
16.5	15	0	0	19
55	39	0	0	46
170	72	4	0.3	82
550	91	30	5	96
2250	94	70	11	97

Percent Transfer of $(^{57}\text{Co})\text{B}_{12}$ from Hog R Protein
to Human Intrinsic Factor by Various Pancreatic
Enzymes

enzyme added (ug/ml)	trypsin (%)	chymotrypsin (%)	elastase (%)	all three (%)
0	0	0	0	0
5.5	0	0	0	0
16.5	0	0	0	0
55	0	0	0	0
170	0	0	0	0
550	1.4	0.7	0	1
2250	13	4	0	5.5

Assays were performed in a volume of 0.45 ml of 0.013 M Tris (Hydroxymethyl) Aminomethane-HCl pH 8.0, 0.187 M NaCl, containing enzyme as indicated, 26.67 ug of bovine serum albumin, and 1 pmol of $(^{57}\text{Co})\text{B}_{12}$ prebound to 1 pmol of R protein. After incubating for 30 minutes at 37°C, 1.5 pmol of Human Intrinsic Factor was added in 0.05 ml and the reaction was incubated for another 30 minutes at 37°C. The samples were then cooled in ice water for 5 minutes and 0.1 ml of rabbit Anti Human Intrinsic Factor anti-serum was added. After an additional 30 minutes, 0.5 ml of H₂O saturated with (NH₄)₂SO₄ was added, followed by centrifugation at 20,000 x g at 4°C for 30 minutes. A portion, 0.5 ml, of the supernatant was then assayed for $(^{57}\text{Co})\text{B}_{12}$. This reflected the amount of $(^{57}\text{Co})\text{B}_{12}$ still bound to R since any $(^{57}\text{Co})\text{B}_{12}$ transferred to Intrinsic Factor was precipitated under the assay conditions.



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Table 2

ABILITY OF CRUDE PANCREATIC ENZYMES TO EFFECT THE TRANSFER OF $(^{57}\text{Co})\text{B}_{12}$
FROM R PROTEIN TO INTRINSIC FACTOR

Percent Transfer of $(^{57}\text{Co})\text{B}_{12}$ from Human R Protein
to Human Intrinsic Factor

solution present# (% of full strength)	Hog pancreatic extract	Human duodenal juice	
		unstimulated	stimulated
0	0	0	0
0.155		0	2
0.225	0		
0.335		0.5	9
0.66	16	4	32
2.20	61		
2.75		15	90
5.50		52	94
6.60	89		
11.0		83	97
22.0	93		
43.5		96	99
50.0	98		
66.0		98	99
86.0	99	99	100



Table 2 Continued

Ability of Crude Pancreatic Enzymes to Effect the Transfer of $(^{57}\text{Co})\text{B}_{12}$

From R Protein to Intrinsic Factor

Percent Transfer of $(^{57}\text{Co})\text{B}_{12}$ from Hog R Protein
to Human Intrinsic Factor

solution present [#] (% of full strength)	Hog pancreatic extract	Human duodenal juice	
		unstimulated	stimulated
0	0	0	0
0.225	0		
0.66	0		
1.30		0	0
2.20	0.3		
5.5		0	3.3
6.60	1.7		
11.0		0	8.7
22.0	9	0	16
33.0			22
44.0		3.7	30
51.0	24		
66.0		10	40
87.0		13	45
90.0	39		

Assays were performed as described in Table 1.

% of full strength = (amount of sample assayed / initial incubation volume) x 100. For example, if 0.2 ml of Human Duodenal Juice was added to this assay the "% of full strength" would equal $\frac{0.2 \text{ ml}}{0.45 \text{ ml}} \times 100\% = 44.4\%$

All volume differences associated with the various amounts of solution present were corrected with 0.15 M NaCl.



hog pancreatic extract was intermediate in this regard. The three solutions had the same relative potencies when tested with hog R protein-B₁₂^{*} (Table 2), but only about 13-46% of the B₁₂^{*} was transferred to human intrinsic factor when they were tested at 89% of their full strength. These studies support the concept that hog R protein-B₁₂^{*} is more resistant to the action of pancreatic enzymes than is human R protein-B₁₂^{*}.

Table 3 shows the results of an experiment in which multiple serial samples of duodenal juice were collected from a normal subject before and after pancreatic secretion was stimulated by the duodenal perfusion of essential amino acids. Trypsin activity and the ability to transfer B₁₂^{*} from R protein to intrinsic factor increased in parallel after pancreatic stimulation. This provides additional evidence for the concept that the transfer of B₁₂ from R protein to intrinsic factor is a specific function of pancreatic enzymes.

It has been found that dog R protein is similar to hog R protein, when it is used in place of hog R protein in experiments similar to those in Tables 1, 2, and 3. It has also been found that rat IF and hog IF are similar to human IF when used in the practice of the present invention.

In the preferred process of the present invention, the subject to be tested has a digestive tract substantially free of undigested food or unprocessed waste materials. This is accomplished, for example, by fasting the subject for about 8 to 12 hours. Then the subject is caused to orally ingest the following, either separately or in combination:

- 1) One labelled form of B₁₂^{**} bound to intrinsic factor (B₁₂^{**}-IF);
- 2) A second labelled form of B₁₂^{*} bound to R protein (B₁₂^{*}-R);
- 3) An unlabelled B₁₂ analogue that binds only to R protein; and
- 4) Free intrinsic factor (IF) (optional).



TABLE 3

CORRELATION OF TRYPSIN ACTIVITY AND THE ABILITY TO TRANSFER (^{57}Co) B_{12}
 FROM R PROTEIN TO IF IN HUMAN DUODENAL JUICE BEFORE AND AFTER
 PANCREATIC STIMULATION

<u>Time after pan- creatic stimulation</u> (minutes)	<u>Trypsin⁺</u> ug/ml	Percent transfer of (^{57}Co) B_{12} from R protein to	
		<u>Human R</u> (%)	<u>Hog R</u> (%)
-10	37	7	0
-5	54	11	0
0	35	1.4	0
+5	97	19	0
+10	272	82	10
+15	360	89	14
+20	271	83	9
+25	189	76	1.7
+30	129	60	0
+35	138	54	0
+40	145	52	0
+45	145	57	0
+50	123	38	0
+55	172	58	0
+60	132	41	0
+65	135	46	0
+70	166	50	0
+75	120	42	0
+80	95	30	0
+85	111	32	0

⁺ Assayed as described in specifications

[‡] Assayed as described in Table 1; 0.020 ml and 0.400 ml of each sample were used for the human R and hog R assays, respectively.



These test solutions were prepared by combining 1 ml of H₂O containing 0.2 nmol of (57Co)B₁₂** (2.5 uCi/nmol) with 0.6 nmol of purified human intrinsic factor present in 4.0 ml of 0.01 M potassium phosphate, pH 7.5, containing 5 0.15 M NaCl and 50 ug/ml of bovine serum albumin. Alternatively, human gastric juice which contains both IF and R protein could be utilized after incubation with suitable B₁₂ analogue, such as cobinamide, B₁₂ (bde-OH) or (3,5,6-Me₃ benzimidazole)B₁₂ in 1 to 1,000,000 fold excess with 10 respect to R protein. These B₁₂ analogues bind to R, but not IF. Thus when (57Co)B₁₂ is subsequently added it is bound only by IF since all of the R is already bound. In a separate tube, 5.0 ml of 0.15 M NaCl containing 0.2 nmol of (58Co)B₁₂* (4.0 uCi/nmol) was combined with 0.3 nmol of 15 R protein present in 5.0 ml of 0.01 M potassium phosphate, pH 7.5, containing 0.15 M NaCl and 50 ug/ml of bovine serum albumin. The IF and R protein solutions were incubated at room temperature for 30 minutes and then cooled to 4°C. Cobinamide, 200 nmol in 1.0 ml of 0.15 M NaCl, was added 20 to the R protein solution and after incubating for 30 minutes at 4°C the intrinsic factor and R protein solutions were combined and stored at -20°C. After thawing, it was found that greater than 95% of the (57Co)B₁₂** and (58Co)B₁₂* were still bound by IF and R protein, respectively, based 25 on assays employing charcoal adsorption and precipitation with specific antisera.

Test solutions were administered orally to subjects who had been fasted for about 8 to 12 hours. An intramuscular injection of 1 mg of nonradioactive (unlabelled) B₁₂ 30 was given at substantially the same time as the oral test solution. Subsequently, about two hours after the test was begun, the subject was allowed to end his fast. The subject's excreted urine was then collected for 24 hours after the administration of the test solution and was 35 assayed for (57Co) and (58Co) with a Beckman Gamma 300 System, Beckman Instruments, Inc., Fullerton, CA.



In one preferred embodiment the effect of pancreatic exocrine function on the ability to absorb B_{12}^* bound to human R protein or hog R protein, versus that of B_{12}^{**} bound to human intrinsic factor, was tested by performing a series 5 of dual label Schilling type tests. Each orally administered test sample contained 0.2 nmol of $(^{58}\text{Co})B_{12}^*$ bound to either human R protein or hog R protein, 0.2 nmol of $(^{57}\text{Co})B_{12}^{**}$ bound to human IF, 0.4 nmol of human IF and 200 nmol of nonradioactive cobinamide, a B_{12} analogue. Cobinamide B_{12} analogue binds to R protein but does not bind with 10 IF. When ingested it serves to prevent endogenous R protein, normally present in the gastric juices, from removing the $(^{57}\text{Co})B_{12}^{**}$ from human IF within the gastrointestinal tract. Nonradioactive B_{12} , 1 mg, was injected intramuscularly in 15 conjunction with the oral ingestion of the test solutions. This injection served to saturate B_{12} -binding proteins in the plasma, thus allowing substantial urinary excretion of absorbed $(^{57}\text{Co})B_{12}^{**}$ and $(^{58}\text{Co})B_{12}^*$ which could then be measured in 24 hour urine collections.

20 The results of these tests when performed on a subject with known pancreatic exocrine insufficiency are shown in Table 4 and are consistent with the in vitro experiments in the following ways: 1) B_{12}^* originating as human R protein- B_{12}^* and hog R protein- B_{12}^* were absorbed less than 25 10% as well as B_{12}^{**} originating as human IF- B_{12}^{**} ; 2) B_{12}^* originating as hog R protein- B_{12}^* was absorbed less well than B_{12}^* originating as human R protein- B_{12}^* ; 3) hog trypsin corrected the malabsorption of B_{12}^* originating as 30 human R protein- B_{12}^* to a much greater extent than it corrected the malabsorption of B_{12}^* originating as hog R protein- B_{12}^* ; and 4) hog pancreatic extract was more potent than hog trypsin in correcting the malabsorption of B_{12}^* originating as hog R protein- B_{12}^* . Because of its greater availability and resistance to pancreatic enzymes, hog R 35 protein was utilized for the remainder of the preferred embodiments presented below.



Table 4

Comparison of the absorption of $(^{58}\text{Co})\text{B}_{12}^*$ bound to Human and Hog R protein (R), versus that of $(^{57}\text{Co})\text{B}_{12}^{**}$ bound to Human intrinsic factor (IF) in dual label pancreatic tests performed on a patient with pancreatic exocrine insufficiency.*

<u>R protein</u>	<u>Addition⁺</u>	Percentage of administered B_{12} excreted in urine		
		<u>IF-B_{12}^{**}</u> (%)	<u>R-B_{12}^*</u> (%)	<u>IF-$\text{B}_{12}^{**}/\text{R-}\text{B}_{12}^*$</u>
Human	None	19.1	1.5	12.7
Hog	None	23.6	0.6	39.3
Human	Trypsin [#]	25.8	19.9	1.3
Hog	Trypsin [#]	16.0	1.8	8.9
Hog	Hog pancreatic extract [#]	14.3	6.3	2.2

* Orally administered solutions (see Methods) contained 0.2 nmol of $(^{58}\text{Co})\text{B}_{12}^*$ bound to Human or Hog R protein, 0.2 nmol of $(^{57}\text{Co})\text{B}_{12}^{**}$ bound to Human IF, enough free Human IF to bind 0.4 nmol of B_{12} , and 200 nmol of nonradioactive cobinamide. The patient was No. 13 per Table 5.

+ Given orally immediately following the solution containing labelled B_{12} .

Crystalline Hog trypsin, 50 mg, freshly dissolved in 50 ml of 0.001M HCl.

Four pancrelipase capsules (KU-ZYME HP).



Following are details of how the relative absorption of B_{12}^{**} originating as human IF- B_{12}^{**} originating as hog R protein- B_{12}^* , serves as a diagnostic test for pancreatic exocrine insufficiency. The results of dual label Schilling type tests employing human IF- B_{12}^{**} and hog R protein- B_{12}^* performed on normal subjects, and patients with pancreatic and other diseases are shown in Table 5. In 11 normal subjects the percent of orally administered B_{12}^{**} in 24-hour urine samples ranged from about 15.4% to 26.7% (mean 21.9%) for B_{12}^{**} originating as IF- B_{12}^{**} and from about 10.0% to 23.1% (mean 17.3%) B_{12}^* for B_{12}^* originating as hog R protein- B_{12}^* . The ratio of urine excreted B_{12}^{**} to B_{12}^* ranged from about 1.0 to 1.8 (mean 1.3). The fact that normal subjects usually absorbed slightly more B_{12}^{**} originating as IF- B_{12}^{**} than B_{12}^* originating as hog R protein- B_{12}^* is consistent with the above in vitro observation, that human pancreatic juice is somewhat limited in its ability to effect the transfer of B_{12}^* from hog R protein to human IF.

In 10 patients with pancreatic disease severe enough to cause steatorrhea (malabsorption of fat), as documented independently by tests for elevated fecal fat contents, values for B_{12}^{**} originating as IF- B_{12}^{**} were close to values of B_{12}^{**} for normal subjects and ranged from about 9.8% to 23.3% (mean 16.5%). The reason for the slight reductions of B_{12}^{**} , originating as IF- B_{12}^{**} , seen in some patients is unknown although they could be due to incomplete urine collections, or minor abnormalities of intestinal pH, bacterial overgrowth, or intestinal mucosa integrity. However, for these same patients the values for B_{12}^* originating as hog R protein- B_{12}^* were uniformly and markedly reduced, ranging from about 0.5% to 2.2% (mean 1.2%). Values for the ratio of urine excreted B_{12}^{**} to B_{12}^* were markedly elevated and ranged from about 6.9 to 46.2 (mean 19.4). The values for B_{12}^* and the ratio of B_{12}^{**} to B_{12}^* did not overlap with the values for any of the normal subjects. The degree of elevation of the ratio of B_{12}^{**} to B_{12}^* appeared



Table 5 (Continued)

Subject #	Age Yrs	Sex	Fecal Rate (E/24 hr)	Disease	Percentage of orally administered B12 In 24 hour urine		
					IF-B12 (%)	R-B12 (%)	IF-E12/R-B12
<u>Patients with pancreatic disease without steatorrhoea</u>							
22	49	H	3	chronic pancreatitis, Etoh, pancreatic calcification, 25%	14.4	5.8	2.5
23	35	H	6	pancreatectomy, moderate fibrosis	30.5	20.9	1.5
24	36	H	2	acute pancreatitis, chronic steroid therapy for asthma	23.0	19.2	1.2
				chronic pancreatitis, Etoh	mean range	(12.6) (13.3) (14.4-10.5) (5.8-20.9)	(1.1-2.5) (1.2-2.5)
<u>Patients with other gastrointestinal diseases</u>							
25	18	F	39	Crohn's disease, mesenteric infarction, 95% am. bowel resection	1.1	0.5	2.2
26	27	F	27	Co cervix, radiation enteritis, bacterial overgrowth	3.9	1.9	2.1
27	60	H		sprue, pernicious anemia	4.6	2.4	1.9
28	68	H		sprue	0.9	0.5	1.8
29	52	F		sprue	5.8	3.7	1.6
30	47	F	8	mesenteric infarction, 2 sm. bowel resection	0.3	0.2	1.5
31	27	F		sprue	12.3	9.5	1.4
32	27	F		pernicious anemia	18.4	11.9	1.5
33	72	F	9	celioderma, fibrodysia, bacterial overgrowth	17.7	14.7	1.2
34	30	H		Crohn's disease, ileal resection	0.7	0.6	1.2
				mean range	(6.7) (4.8) (0.3-16.4) (0.2-14.7)	(1.1-6) (1.2-2.2)	
<u>Patients with pancreatic disease and other gastrointestinal diseases</u>							
35	57	H		chronic pancreatitis, pseudocyst, Crohn's disease, ileal resection	2.3	0.4	5.8
36	45	H	19	pseudocyst, Whipple procedure, 90% pancreatectomy, sprue	4.3	0.2	21.5
<u>Patients with pancreatic disease and renal insufficiency</u>							
37	54	F	40	chronic pancreatitis, Etoh, pancreatic calcification, renal insufficiency (creatinine clearance 6 ml/min)	5.7	1.3	4.4
38	61	P		adenocarcinoma pancreas, Whipple procedure, total pancreatectomy, renal insufficiency (creatinine clearance 20 ml/min)	6.6	0.3	22.0

Finally administered solutions were the same as those used in Table 3 except that in 20 subjects 1, 5, 6, 10, 12, 18, 17, 21, 27, 32, 33 and 35 the radioactive forms of B12 were reversed such that [⁵⁷Co]B12 was given bound to IF and [⁵⁷Co]B12 was given bound to R.

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Table 5
Results of dual label pancreatic tests performed on
normals, and patients with pancreatic and other gastrointestinal diseases*

Subject #	Age yr	Sex	Fecal Fat (g/24 hr)	Disease:				Percentage of orally administered Bi2 in 24-hour urine IF-Bi2 R-Bi2 IF-Bi2/R-Bi2 (%)
				IF-Bi2	R-Bi2	IF-Bi2 R-Bi2	(%)	
<u>Normal Subjects</u>								
1	53	F		None		26.9	11.8	
2	24	F		"		16.4	10.0	1.6
3	28	F		"		26.7	19.3	1.4
4	35	H		"		21.0	16.8	1.4
5	62	H		"		13.4	12.1	1.3
6	34	F		"		23.6	20.1	1.3
7	31	H		"		23.2	19.8	1.2
8	29	H		"		16.9	16.4	1.2
9	26	F		"		23.0	18.8	1.2
10	32	H		"		22.9	20.5	1.1
11	40	H		"		22.1	21.0	1.0
				mean		(21.9)	(17.3)	(1.3)
				range		(15.6-26.7)	(10.0-23.1)	(1.0-1.8)
				mean \pm 2 S.D.		(14.4-29.4)	(9.0-25.6)	(0.7-1.9)
<u>Patients with pancreatic disease and steatorrhoea</u>								
12	66	P	4.7	adenocarcinoma pancreas, Whipple procedure, 100% pancreatectomy		21.1	0.5	46.2
13	54	N	15.1	chronic pancreatitis, idiopathic, pancreatic calcification		21.6	0.6	39.3
14	46	H	39	chronic pancreatitis, Etoh, 90% pancreatectomy		18.4	0.9	20.4
15	18	H	51	cystic fibrosis		13.6	0.7	19.4
16	67	H	18	chronic pancreatitis, Etoh, indurated pancreas at laparotomy		18.1	1.1	17.0
17	67	H	12	chronic pancreatitis, Etoh, pancreatic calcification		14.0	1.2	11.7
18	38	H	18	chronic pancreatitis, Type IV hyperlipidemia		11.6	1.0	11.6
19	52	H	27	chronic pancreatitis, Etoh, pancreatic calcification		17.3	1.6	10.8
20	58	H	27	chronic pancreatitis, Etoh, pancreatic calcification		9.8	0.9	10.7
21	26	F	19	gun shot wound, Whipple procedure, 75% pancreatectomy		15.1	2.2	5.9
				mean		(16.5)	(11.1)	(19.9)
				range		(9.8-23.6)	(0.5-2.2)	(6.9-46.2)



to be in agreement with the severity of steatorrhea since the 5 patients with the highest ratios were the same 5 patients that also had the highest fecal fat contents.

While correlation was not perfect, this may have been due 5 to the fact that the patients were not on diets of defined fat content while feces were being collected for the fecal fat tests.

Using the test of the present invention, two other patients with pancreatic disease that did not cause stea- 10 torrhea had normal test results while a third patient with a pancreatic disease had a slightly reduced value of 5.8% for B_{12}^* and a slightly increased value of 2.5 for the ratio of B_{12}^{**} to B_{12}^* . These results suggest that the tests of the present invention provide similar, and possibly somewhat 15 superior results to the 72-hour fecal fat analysis in terms of its sensitivity in detecting patients with pancreatic exocrine insufficiency.

The specificity of the test of the present invention was assessed by performing it on 10 patients with a variety 20 of gastrointestinal diseases that included Crohn's disease, sprue, small bowel resection, scleroderma, bacterial over-growth, giardiasis and pernicious anemia. Vitamin B_{12} malabsorption is frequently present in many of these diseases and values for B_{12}^{**} originating as 'IF- B_{12}^{**} ranged 25 widely from about 0.3% to 18.4% (mean 6.7%) in these pa-tients. Values for B_{12}^* originating as R- B_{12}^* were reduced proportionately, however, and ranged from about 0.2% to 14.7% (mean 4.8%). This resulted in values for the ratio B_{12}^{**} to B_{12}^* that ranged from about 1.2 to 2.2 (mean 1.6), 30 which were very similar to the ratios of normal subjects. Values for the B_{12}^{**} to B_{12}^* ratio in these subjects with gastrointestinal disease did not overlap with those ob-tained for patients with pancreatic disease severe enough to cause steatorrhea.

35 It is of interest to note the fact that 4 patients within the intestinal disease group had documented stea-



torrhea. This demonstrates that steatorrhea itself is not responsible for elevated values for the ratios of B_{12}^{**} to B_{12}^* .

One patient with both pancreatic disease and Crohn's disease, and a second patient with both pancreatic disease and sprue, were studied to determine if the test could detect pancreatic exocrine insufficiency in the face of superimposed intestinal disease. In the first patient, with Crohn's disease, the value for B_{12}^{**} originating as IF- B_{12}^{**} was reduced to 3.2%, but the value for B_{12}^* originating as R- B_{12}^* was reduced even further to 0.4% thus giving an elevated value of 5.8 for the ratio of B_{12}^{**} to B_{12}^* . In the second patient, with sprue, the value for B_{12}^{**} was reduced to 6.6% but the value for B_{12}^* was reduced disproportionately to 0.3% thus giving an elevated value of 22.0 for the ratio of B_{12}^{**} to B_{12}^* . These results indicate that the test of the present invention is capable of detecting pancreatic exocrine insufficiency even in the presence of superimposed intestinal disease.

Two patients with combined pancreatic and renal disease were studied, since it is known that the urinary excretion of B_{12} is reduced in patients with renal disease. Both patients had low values for B_{12}^{**} originating as IF- B_{12}^{**} , 5.7% and 6.6% respectively, but their values for B_{12}^* originating as R- B_{12}^* were reduced disproportionately to 1.3% and 0.3% respectively. Consequently, their values for the ratio of B_{12}^{**} to B_{12}^* of 4.4 and 22.0 respectively, were both elevated. These results indicate that the test of the present invention can detect pancreatic exocrine insufficiency in patients with superimposed renal disease provided that such patients are not anuric.

Using the equivalent of the present invention, plasma levels of absorbed B_{12} can be monitored and are especially useful in patients who are anuric. Plasma levels could also be used in subjects without renal disease, as has been done with standard B_{12} absorption tests, although here the



injection of unlabelled B_{12} would be omitted since such omission increases plasma levels of recently absorbed labelled B_{12} .

In most of the studies presented in Table 5, $(^{57}Co)B_{12}^{**}$ was given bound to intrinsic factor and $(^{58}Co)B_{12}^*$ was given bound to hog R protein. However, the labelling isotopes were reversed, in at least several subjects in each major group and this did not appear to influence the results of the test.

It has been determined that the time course of the urinary excretion of B_{12}^{**} and B_{12}^* is not critical. The time course of the urinary excretion of B_{12}^{**} originating as IF- B_{12}^{**} and B_{12}^* originating as R- B_{12}^* for one patient with pancreatic exocrine insufficiency and one normal subject are presented in Table 6. The data indicate that in both cases the ratio of B_{12}^{**} to B_{12}^* was highest in the 0 to 8 hour samples and lowest in the 16 to 24 hour samples. However, differences were slight, during these time periods, and this suggests that the additional time required for pancreatic enzymes to act on B_{12}^* originating as hog R- B_{12}^* and effect the transfer of such B_{12}^* to IF is small compared with the time required for such B_{12}^* to reach the ileum and be absorbed. Because the ratio differences at various times were small, the inadvertent loss of individual urine samples during a 24-hour collection would not be expected to have a major effect on the value obtained for the ratio. The data presented in Table 6 also indicate that one could utilize 0 to 8 hour urine collections to evaluate patients for pancreatic exocrine insufficiency if time was a critical factor.

The model upon which the present invention is based can also serve to explain certain anomalies noted in situations of B_{12} absorption in certain cases of pancreatic exocrine dysfunction. Vitamin B_{12} malabsorption in pancreatic insufficiency has been corrected by the oral administration of bicarbonate, pancreatic proteases or the B_{12}



Table 6

Time course of urinary excretion of radioactivity after the simultaneous oral administration of Human intrinsic factor (IF)-(⁵⁷Co)B₁₂^{**} and Hog R protein-(⁵⁶Co)B₁₂* in pancreatic tests.*

<u>Subject</u>	Time after oral administration (Hr)	Percentage of administered B ₁₂ excreted in 24 hour urine		
		IF-B ₁₂ (%)	R-B ₁₂ (%)	IF-B ₁₂ /R-B ₁₂
Patient with pancreatic exocrine insufficiency ⁺	0 - 8	8.52	0.45	18.9
	8 - 16	5.02	0.27	18.6
	16 - 24	0.060	0.004	15.0
	0 - 24	13.600	0.724	18.9
Normal [‡]	0 - 8	4.30	3.53	1.22
	8 - 16	10.59	9.30	1.14
	16 - 24	3.97	3.53	1.12
	0 - 24	18.86	16.36	1.15

*Orally administered solution were the same as those used in Table 4.

[†]The patient was No. 15 per Table 5.

[‡]Subject No. 8 per Table 5.



analogue cobinamide. The model on which the present invention is based provides an explanation for the action of these seemingly diverse B_{12} malabsorption correcting agents. Bicarbonate exerts its effect by neutralizing gastric acid
5 since at neutral (non-acid) pH IF can partially compete with R protein for the initial binding and retention of B_{12} , thus providing IF- B_{12} for absorption. Pancreatic proteases function by partially degrading R protein and lowering its affinity for B_{12} by 150-fold, and these changes result in
10 the rapid transfer of B_{12} from R protein to IF, again providing IF- B_{12} for absorption. Cobinamide B_{12} analogue binds to R protein with high affinity, but does not bind to IF. This B_{12} analogue functions by saturating endogenous R protein and leaving IF as the only binder available for
15 binding B_{12} , thus again providing IF- B_{12} for absorption.

The model on which the present invention is based also provides an explanation for the fact that many patients with severe pancreatic exocrine insufficiency have normal Schilling tests since achlorhydria has the same effect as oral
20 bicarbonate and is frequently superimposed with this exocrine insufficiency, and also since some variation does exist in the R protein content of basal gastric juice.

The data presented herein provides support for the present B_{12} absorption model and the test of the present
25 invention. It shows that B_{12}^* given orally bound to R protein was malabsorbed markedly by all 10 subjects studied with pancreatic exocrine insufficiency severe enough to cause steatorrhea. Simultaneously administered B_{12}^{**} given bound to IF, and given with cobinamide B_{12} analogue resulted in the B_{12}^{**} being absorbed in essentially normal amounts, by all of these same subjects. This observation provides strong evidence against the concept that pancreatic proteases alter the structure of IF in a manner that is required for B_{12} absorption. Preferential malabsorption
30 of B_{12}^* originating as R- B_{12}^* was not observed at all in a variety of intestinal diseases. This indicates that the
35



abnormalities in B_{12} malabsorption frequently seen with intestinal diseases are not due to interference with the partial degradation of R protein by pancreatic proteases or with the transfer of B_{12}^* to intrinsic factor, even

5 though these steps do take place within the intestine.

The dual label test of the present invention is faster than the 72-hour fecal fat analysis and is more convenient and less disagreeable for the subject and laboratory personnel than either duodenal intubation tests or the fecal 10 fat analysis. The present test also possesses the potential of being much more easily available than either of the two current tests for pancreatic exocrine function since it could be performed by any physician with access to a rudimentary nuclear medicine facility, provided that the 15 test components were available.

The tests of the present invention are specific for pancreatic exocrine insufficiency. Such specificity is not possessed by the fecal fat analysis, since this test is also abnormal in a variety of intestinal diseases. Specificity is important clinically since weight loss, diarrhea, and abdominal pain are common symptoms in both pancreatic and intestinal diseases. Specificity is also important in evaluating patients with known intestinal disease for the presence of superimposed pancreatic exocrine insufficiency.

25 Although the ratio of B_{12}^{**} originating as IF- B_{12}^{**} to B_{12}^* originating as hog R- B_{12}^* is the parameter which provides the test of the present invention with its specificity for pancreatic exocrine insufficiency, it should be noted that useful information is also provided by the values obtained for B_{12}^{**} itself since a marked reduction in B_{12} was often observed in patients with intestinal disease. However, as marked reductions for B_{12}^{**} were not always observed, a normal value for IF- B_{12}^{**} does not rule out the presence of intestinal disease.

35 Although most of the studies presented here utilized



human IF, hog R protein, cobinamide B₁₂ analogue, (⁵⁷Co)B₁₂, and (⁵⁸Co)B₁₂, it is well established by the studies presented here and elsewhere that similar results could be obtained by substitution of equivalent compositions for these components. For example, other species of IF, such as hog IF, rat IF, and others could be used in place of or in combination with human IF. Other R proteins or other materials which bind B₁₂ and make it unavailable for binding to IF in the digestive system, unless pancreatic enzymes are present, could be used with or in place of hog R protein. These include human R protein and dog R protein and any other material with these properties.

Several techniques, other than the use of B₁₂ analogues exist for preventing endogenous R protein from removing B₁₂ from IF in the digestive system. One method would employ B₁₂ analogues in place of or in combination with cobinamide B₁₂ analogue, which other B₁₂ analogues also possess the property of binding to human R protein with substantially the same affinity as B₁₂ while binding with low affinity to IF. B₁₂ (bde-OH) and (3,4,6-Me₃ benzimidazole)B₁₂ are examples of some other B₁₂ analogues which possess these properties. Another method of preventing R protein from removing B₁₂ from IF in the digestive system would provide for the effective removal or neutralization of acid in the subject's stomach, since at neutral or slightly alkaline pH R protein is much less effective in removing B₁₂ from IF than it is at acid pH. The acid could be neutralized by the oral administration of bicarbonate or other antacids or by the oral or parenteral administration of agents, such as the drug cimetidine, which limit the secretion of acid into the gastric juices. Many labelled forms of vitamin B₁₂ are available for use in the practice of the present invention, such as (⁵⁶Co)-B₁₂ or (⁶⁰Co)-B₁₂ which could be utilized in place of (⁵⁷Co)-B₁₂ and (⁵⁸Co)-B₁₂.

Although the studies presented here measure the ab-



sorption of various labelled forms of B₁₂ with the Schilling technique, it is well established that similar results could be obtained by using other methods of measuring the absorption of labelled B₁₂. Examples of such applicable techniques include measuring labelled B₁₂ in feces or blood or by measuring the total body content or hepatic content of radioactive B₁₂ with nuclear scanning.

Although the studies presented here utilize two different labelled forms of B₁₂ so that the absorption of B₁₂ originating in both IF-B₁₂ and R-B₁₂ could be measured simultaneously, it is apparent that similar results could be obtained by using one labelled form of B₁₂ and measuring the absorption of IF-B₁₂ and R-B₁₂ on separate occasions.

While preferred embodiments have set forth concentrations and amounts of various components utilized in certain studies, the useful and preferred amounts of those components are set forth below:

<u>Composition</u>	<u>Useful Range</u>	<u>Preferred Range</u>
IF-B ₁₂	0.002-20.0 nmol	0.05-2.0 nmol
R-B ₁₂	0.002-20.0 nmol	0.05-2.0 nmol
Free IF	0.05-100.0 nmol	0.2-1.0 nmol
B ₁₂ analogues	0.2-20,000 nmol	20-500 nmol

While the invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that the foregoing and other modifications or changes in form and detail may be made therein without departing from the spirit and scope of the invention.

What is claimed is:



CLAIMS

30.

1. In the method of testing pancreatic exocrine function of a subject the steps of:
placing in the subject's digestive system a complex of first labelled vitamin B₁₂ bound to intrinsic factor protein (IF) said complex being designated as IF-B₁₂;
and
also placing in the subject's digestive system a composition including second labelled vitamin B₁₂ bound to binding means, wherein said binding means bind with said second labelled B₁₂, and wherein said binding means substantially inhibit the binding of said second labelled B₁₂ by IF in said subject's digestive system until said composition is acted upon by exocrine pancreatic enzymes.
- 15 2. The method of claim 1 wherein said subject has endogenous R protein in his digestive system and said subject is caused to ingest R protein blocking means for rendering said endogenous R protein substantially incapable of removing said first labelled B₁₂ from said IF-B₁₂ complex.
3. The method of claim 1 wherein said subject is caused to ingest unbound intrinsic factor protein.
4. The method of claim 1 wherein said IF is selected from the group consisting of human intrinsic factor, hog intrinsic factor and rat intrinsic factor.
- 25 5. The method of claim 4 wherein said IF is human intrinsic factor.
6. The method of claim 1 wherein the binding means for said second labelled vitamin B₁₂ is an R protein and said composition is an R-B₁₂ complex.
- 30 7. The method of claim 6 wherein said binding R protein is selected from the group consisting of human R protein, dog R protein, and hog R protein.
8. The method of claim 7 wherein the binding R protein is hog R protein.
- 35 9. The method of claim 2 wherein said R protein blocking



means is a B_{12} analogue which reacts with and substantially binds R protein, but which does not bind substantially with IF protein.

10. The method of claim 9 wherein said B_{12} analogue is selected from the group consisting of cobinamide, $B_{12}(\text{bde-OH})$ and $(3,5,6-\text{Me}_3\text{benzimidazole})B_{12}$.
11. The method of claim 10 wherein said B_{12} analogue is cobinamide.
12. The method of claim 2 wherein said R protein blocking means removes acid from the subject's stomach.
13. The method of claim 12 wherein said acid removing means is selected from the group consisting of antacids and means that limit secretion of acid into the digestive system.
14. The method of claim 13 wherein the antacid is sodium bicarbonate.
15. The method of claim 13 wherein the means for limiting acid secretion is cimetidine.
16. The method of claim 1 wherein the first and second labelled B_{12} are selected from the group consisting of $(^{56}\text{Co})B_{12}$, $(^{57}\text{Co})B_{12}$, $(^{58}\text{Co})B_{12}$ and $(^{60}\text{Co})B_{12}$.
17. The method of claim 1 including the additional step of injecting the subject with unlabelled B_{12} in an amount sufficient to react with and bind substantially all B_{12} binding substances present in the subject's blood and plasma.
18. The method of claim 17 wherein the subject's excreted urine is subsequently collected and measured to determine the amount of first labelled B_{12} and the amount of second labelled B_{12} which were absorbed and subsequently excreted.
19. The method of claim 1 wherein said first labelled B_{12} and said second labelled B_{12} are labelled differently from one another.
- 35 20. The method of claim 1 wherein the IF- B_{12} complex is ingested in an amount in the range of about 0.002 to about 20.0 nmol.



21. The method of claim 1 wherein the IF-B₁₂ complex is ingested in an amount in the range of about 0.05 to about 2.0 nmol.
22. The method of claim 1 wherein the IF-B₁₂ complex is ingested in an amount of about 0.2 nmol.
5. 23. The method of claim 6 wherein the R-B₁₂ complex is ingested in an amount in the range of about 0.002 to about 20.0 nmol.
10. 24. The method of claim 6 wherein the R-B₁₂ complex is ingested in an amount in the range of about 0.05 to about 2.0 nmol.
25. The method of claim 1 wherein the R-B₁₂ complex is ingested in an amount of about 0.2 nmol.
15. 26. The method of claim 9 wherein said B₁₂ analogue is ingested in an amount in the range of about 0.2 to about 20,000 nmol.
27. The method of claim 9 wherein said B₁₂ analogue is ingested in an amount in the range of about 20 to about 500 nmol.
20. 28. The method of claim 9 wherein said B₁₂ analogue is ingested in an amount of about 200 nmol.
29. The method of claim 3 wherein the unbound intrinsic factor is ingested in an amount in the range of about 0.05 to about 100 nmol.
25. 30. The method of claim 3 wherein the unbound intrinsic factor is ingested in an amount in the range of about 0.2 to about 1.0 nmol.
31. The method of claim 3 wherein the unbound intrinsic factor is ingested in an amount of about 0.4 nmol.
30. 32. The method of testing pancreatic exocrine function of a subject including the steps of:
causing the subject to ingest into his digestive system a complex of B₁₂-IF, wherein said B₁₂ is labelled and is selected from the group consisting of radioactive (56Co)B₁₂, (57Co)B₁₂, (58Co)B₁₂ and (60Co)B₁₂;
35 causing the subject to ingest a composition including



second labelled vitamin B₁₂ bound to hog R protein, wherein said second labelled B₁₂ is different from said first labelled B₁₂ and is selected from the group consisting of radioactive (57Co)B₁₂, (58Co)B₁₂, (59Co)B₁₂ and (60Co)B₁₂;

5 causing the subject to ingest endogenous R protein blocking means in the form of B₁₂ analogue;

causing the subject to ingest bound intrinsic factor;

injecting said subject with B₁₂;

10 subsequently collecting said subject's excreted urine; and then

measuring said excreted urine to determine the amount of said first labelled radioactive B₁₂ and the amount of said second labelled radioactive B₁₂.

15 33. A composition for use in testing pancreatic exocrine function and containing, in combination;

a complex of IF and labelled B₁₂;

a complex of R protein and labelled B₁₂; and

B₁₂ analogue capable of binding substantially with

20 R protein but not capable of binding substantially with IF.

34. The composition of claim 33 including, in addition, free intrinsic factor.

35. The method of providing a complex of labelled B₁₂

25 and intrinsic factor including the steps of obtaining gastric juices containing both IF and R protein; adding R protein binding material to said juices in an amount sufficient to bind substantially all of said R protein, said R protein binding material binding substantially with R protein and not binding sub-

30 stantially with IF; and

adding labelled B₁₂ to said mixture either at the time that the R binding material is added or after said R binding material is added.

35 36. The method of claim 35 wherein said R protein binding material is a B₁₂ analogue and wherein it is present



in an amount in the range of about 1 to about 1,000,000 times the amount of R protein in the sample.

37. The method of claim 36 wherein said B₁₂ analogue is selected from the group consisting of cobinamide, B₁₂(bde-OH) and (3,4,6-Me₃benzimidazole)B₁₂.

5 38. The labelled B₁₂-IF produced by the process of claim 36.

39. The method of claim 1 including the additional step of measuring the amount of labelled B₁₂ in the subject's blood or plasma.

10 40. The method of claim 1 including the additional step of measuring the amount of labelled B₁₂ in the subject's excreted feces, and then determining the amount of labelled B₁₂ which has been absorbed as the difference between the amounts of labelled B₁₂ which was ingested and the amount of labelled B₁₂ which is excreted in the feces.

15 41. The method of claim 1 wherein the subject is allowed to excrete all unabsorbed labelled B₁₂, wherein said labelled B₁₂ is radioactive, and wherein the amount of radioactivity retained in the subject's body is measured as an indication of absorbed B₁₂.

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I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all):

According to International Patent Classification (IPC) or to both National Classification and IPC *Wo 80/01415*
INT C13 G 01 N 33/50, A 61 K 31/68, 43/00, 49/00, C 07 G 7/00
US Cl. 424/1, 1.5, 9, 12, 201, 359; 128/659; 23/230B

II. FIELDS SEARCHEDMinimum Documentation Searched⁴

Classification System	Classification Symbols
US	424/1, 1.5, 9, 12, 201, 359 23/230B 128/659

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁵

III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴

Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	US, A, 3,786,140 Published 15 January, 1974, Meyer-Bertenrath et al.	1-41
A	US, A, 3,903,253, Published 2 September, 1975.	1-41
X, P	US, A, 4,133,951 Published 9 January, 1979.	1-41
X	US, N, Chemical Abstracts, Issued January, 1978, Abstract Number 451q, Von der Lippe et al.	1-41

* Special categories of cited documents:¹⁶

"A" document defining the general state of the art

"E" earlier document but published on or after the International filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATIONDate of the Actual Completion of the International Search²

22 April 1980

Date of Mailing of this International Search Report²

02 MAY 1980

International Searching Authority¹

ISA/US

(Signature of Authorized Officer¹⁰)*C. M. Nucker*

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